An ABC Transporter Is Required for Secretion of Peptide Sex Pheromones in Enterococcus faecalis

RESEARCH ARTICLE

An ABC Transporter Is Required for Secretion of Peptide Sex Pheromones in *Enterococcus faecalis*

Sriram Varahan,[a] Nathan Harms,[b] Michael S. Gilmore,[c] John M. Tomich,[d] Lynn E. Hancock[a]

Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas, USA[a]; Division of Biology, Kansas State University, Manhattan, Kansas, USA[b]; Departments of Ophthalmology and Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA[c]; Department of Biochemistry, Kansas State University, Manhattan, Kansas, USA[d]

ABSTRACT **Enterococci are leading causes of hospital-acquired infection in the United States and continue to develop resistances to new antibiotics. Many *Enterococcus faecalis* isolates harbor pheromone-responsive plasmids that mediate horizontal transfer of even large blocks of chromosomal genes, resulting in hospital-adapted strains over a quarter of whose genomes consist of mobile elements. Pheromones to which the donor cells respond derive from lipoprotein signal peptides. Using a novel bacterial killing assay dependent on the presence of sex pheromones, we screened a transposon mutant library for functions that relate to the production and/or activity of the effector pheromone. Here we describe a previously uncharacterized, but well-conserved, ABC transporter that contributes to pheromone production. Using three distinct pheromone-dependent mating systems, we show that mutants defective in expressing this transporter display a 5- to 6-order-of-magnitude reduction in conjugation efficiency. In addition, we demonstrate that the ABC transporter mutant displays an altered biofilm architecture, with a significant reduction in biofilm biomass compared to that of its isogenic parent, suggesting that pheromone activity also influences biofilm development. The conservation of this peptide transporter across the *Firmicutes* suggests that it may also play an important role in cell-cell communication in other species within this important phylum.**

IMPORTANCE ***Enterococcus faecalis* ranks as one of the leading causes of hospital-associated infections. Strains possessing resistance to multiple antibiotics are becoming all too common in clinical settings. Pheromone-responsive plasmids play an important role in harboring and disseminating these antibiotic resistance genes. Here we have identified a novel ABC transporter that is responsible for the secretion of peptide pheromones, which enables communication between cells to mediate plasmid transfer. We have also shown that this transporter is important for biofilm formation, providing a strong rationale for its use as a viable therapeutic target which could be targeted to curb infection, as well as the spread of existing drug resistance.**

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Address correspondence to Lynn E. Hancock, lynnh@ku.edu.

Enterococcus faecalis is an opportunistic pathogen that has emerged as a leading cause of hospital-acquired infection in the United States (1). Enterococci exhibit resistance to many antibiotics and heavily colonize patients after antibiotic therapy (2). *E. faecalis* is particularly facile at exchanging mobile elements, having evolved a pheromone-mediated conjugation system (3). Recipient cells release an array of pheromones into the environment, and a potential donor specifically responds to a unique pheromone by inducing a conjugative mating response that includes the production of a plasmid-encoded adhesive protein called "aggregation substance" that promotes effective mating pair formation in liquid cultures, leading to efficient plasmid transfer from donor to recipient (4–8).

Distinct pheromone-responsive plasmid systems have been identified in *E. faecalis* (9) and are known to harbor traits that contribute to the severity of infection, including cytolysin, aggregation substance, and a wide variety of antibiotic resistance genes (10–12). Peptide pheromones derive from lipoprotein signal peptides and are generally heptamer or octamer peptides comprised of predominantly hydrophobic amino acids (13). Eep, a zinc-dependent membrane metalloprotease, has been shown to be involved in processing the lipoprotein precursor, leading to production of the active pheromone in most but not all pheromone systems (14, 15). A recent study (M. S. Gilmore, M. Rauch, M. Ramsey, P. Himes, S. Varahan, F. Lebreton, and L. E. Hancock, submitted for publication) showed that production of a specific pheromone, cOB1, by the commensal *E. faecalis* in the gastrointestinaltractconsortiuminhibitsthevancomycin-resistanthospital-adapted *E. faecalis* strain V583. A transposon screen to identify additional functions involved in this peptide-mediated antagonism led to the identification of mutants that were defective in inhibiting V583. A series of independent transposon mutants whose insertion localized to well-conserved genes encoding a predicted ABC transporter that belongs to the EcsAB family (16) were identified. It was therefore of interest to further characterize this mutant to determine the extent to which the well-conserved transporter contributes to generalized pheromone production in *E. faecalis*. We have designated this ABC transporter the peptide

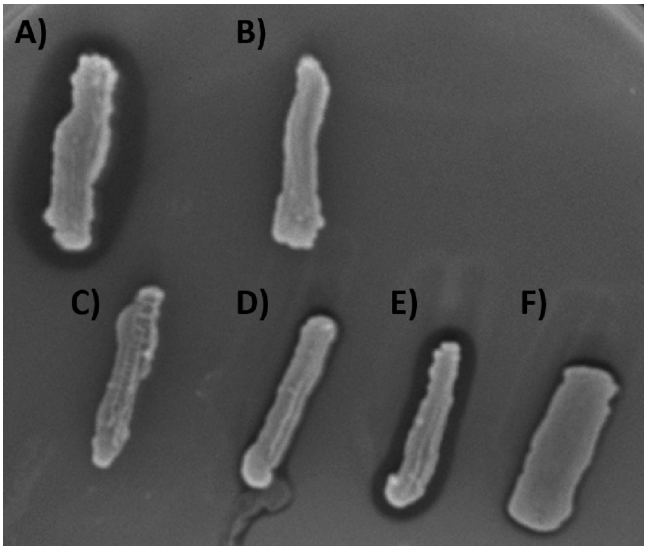

FIG 1 Growth inhibition assay. (A) FA2-2 parental strain; (B) FA2-2::EF_0688; (C) OG1RF::EF_0689a; (D) OG1RF::EF_1723; (E) OG1RF parental strain; (F) OG1RF::EF_0689b. Strains were streaked onto THB agar and grown overnight at 37°C, and then the indicator strain V583 was added in a soft agar overlay. Zones of clearance around the strains are indicative of the growth inhibition of V583.

pheromone transporter (PptAB) to reflect its global role in peptide secretion.

RESULTS

A transposon screen identified mutants with altered activities in inhibiting V583. With the goal of identifying factors that contributed to the pheromone-mediated killing of V583, a transposon mutagenesis screen was performed to identify genes whose protein products contribute to the inhibition of *E. faecalis* V583. A recent study by Hancock et al. (submitted) identified the peptide pheromone cOB1 as being responsible for the growth inhibition of strain V583. We anticipated that mutants discovered in such a screen would identify factors important in the synthesis of the cOB1 peptide pheromone. To address possible strain differences that might contribute to pheromone-mediated killing, we subjected *E. faecalis* strains FA2-2 and OG1RF to transposon mutagenesis using the Himar transposon in plasmid pCAM45 (17). Previous studies with OG1RF demonstrated a role for GelE in the turnover of peptide pheromones (18), and strain FA2-2 possesses a deletion that encompasses most of the *fsr* quorum-sensing system and renders it less proteolytic than OG1RF (19). Transposon mutants from both strains were screened for the ability to inhibit the growth of strain V583. In total, ~30,000 mutants were screened from both libraries, and 10 unique transposon insertions localizing to 7 predicted gene products were identified as displaying defects in peptide-mediated killing of V583 (Fig. 1 and see Table S1 in the supplemental material). Predictably, we identified a transposon insertion in the OG1RF library that localized to EF_1723, a predicted lipoprotein signal peptidase (LspA) thought to be responsible for cleaving lipoproteins at the conserved cysteine in the lipobox domain (20). Additional mutants were identified with insertions in EF_0252, a predicted CHAP domain-containing amidase, a cation transporter (EF_2556), and two predicted hypothetical proteins (EF_2470 and EF62_1081). Our attention was focused on insertions that were confined to a predicted ABC transporter, as independent transposon insertions localized to either the putative ATP-binding cassette (EF_0688) or the predicted permease (EF_0689) of the ABC transporter in both the FA2-2 and OG1RF transposon libraries. EF_0688 shares 62% sequence identity (82% sequence similarity) with *Bacillus subtilis* EcsA, a protein predicted to contain an ATP-binding cassette. EF_0689 shares 30% sequence identity (52% sequence similarity) with the *B. subtilis* EcsB protein, a predicted ABC transporter permease. In *B. subtilis*, EcsAB has been shown to play an important role in exoenzyme secretion (E), competence (C), and sporulation (S) (16).

Disruption of the permease encoded by EF_0689 does not alter exoprotease production in *E. faecalis*. *E. faecalis* is not known to be naturally competent and lacks the sporulation machinery. We tested whether this transporter was involved in exoenzyme secretion in *E. faecalis*, as an *ecsAB* mutation in *Staphylococcus aureus* also resulted in changes to its secretome (21). Since strain OG1RF possesses a functional Fsr system and actively secretes the only known exoproteases (gelatinase and serine protease) in a quorum-dependent manner, we examined the proteolytic activities of the parental strain and the two independent transposon insertion mutants with the *ecsB* homolog EF_0689, which we now refer to as *pptB*. As noted in Fig. S1 in the supplemental material, there was no apparent change in the levels of proteolytic activity between the strains, suggesting that the ABC transporter does not affect exoprotease secretion in *E. faecalis*. In contrast, the known Fsr-deficient strain FA2-2 produces basal protease activity on skim milk agar.

As previously stated, gelatinase has been shown to cleave peptide pheromones, and this activity reduces the mating efficiency between donor and recipient strains (18). In addition, gelatinase is also responsible for the proteolytic activation of the major autolysin, AtlA, and this leads to targeted lysis of bystander cells in a process termed fratricide (22). To both confirm the transposon mutant phenotype and generate a markerless mutation, we generated an in-frame deletion of both EF_0688 (*pptA*) and EF_0689 (*pptB*) in the FA2-2 strain background to minimize the contribution of gelatinase in subsequent assays. This mutant was designated FA2-2Δ*pptAB*. Additionally, we created a complemented strain in which *pptAB* was genetically restored to its native locus, using a gene knock-in strategy (23), and this strain was designated FA2-2Δ*pptAB*::*pptAB*. We compared the levels of growth of the FA2-2 parental strain, the *pptAB* deletion mutant, and the complemented strain in Todd-Hewitt broth and found that the growth of all three strains were nearly identical throughout the growth cycle (see Fig. S2 in the supplemental material).

cOB1 is not detected in the culture supernatant of the *pptAB* deletion mutant. To confirm that the lack of inhibition of V583 by the FA2-2Δ*pptAB* strain was due to the absence of secreted cOB1, we analyzed the culture supernatants from both FA2-2 and the *pptAB* deletion mutant for the presence of cOB1. Strains were grown in complete defined medium (CDM) (24) to reduce the complexity of detecting a short hydrophobic peptide in rich lab medium. Spent culture supernatants from overnight cultures were harvested for high-performance liquid chromatography (HPLC) analysis to identify the presence of cOB1. As expected, we were unable to detect cOB1 in the supernatant from the *pptAB* mutant, whereas detectable cOB1 signal was observed in the parental FA2-2 strain (Fig. 2).

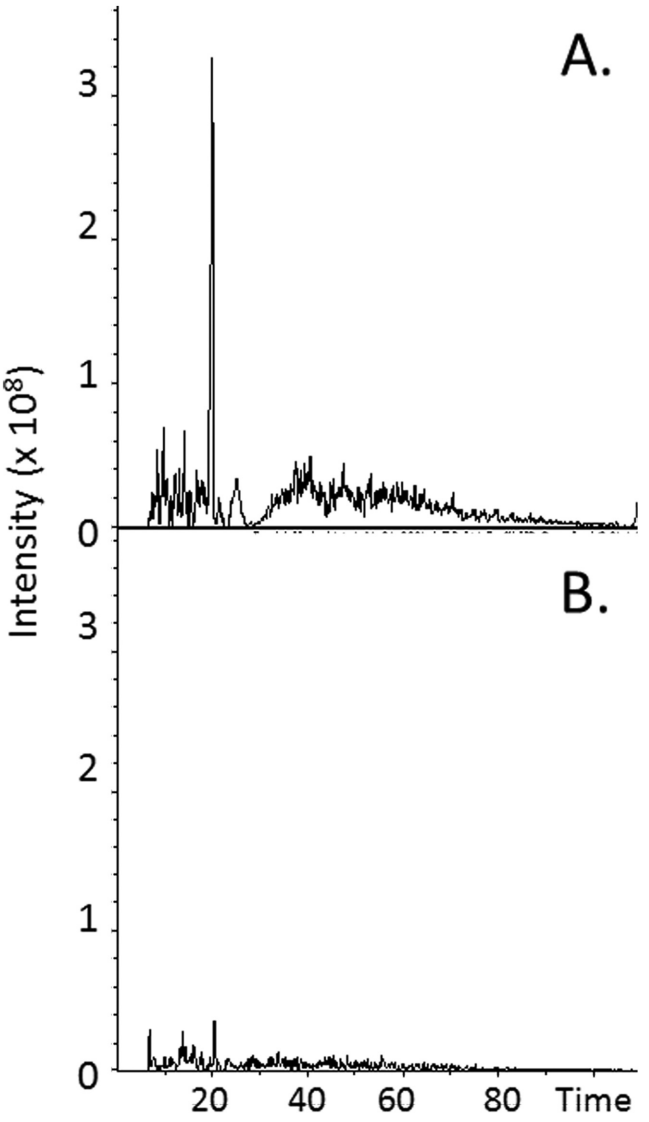

FIG 2 Analysis of the supernatant to identify cOB1. FA2-2 (A) and FA2-2Δ*pptAB* (B) were grown in chemically defined medium (CDM) overnight. Spent culture supernatants were analyzed by chromatography (see the supplemental materials and methods [Text S1]). The peak shows the amount of cOB1 eluted between 17 and 19 min. The time scale is in minutes.

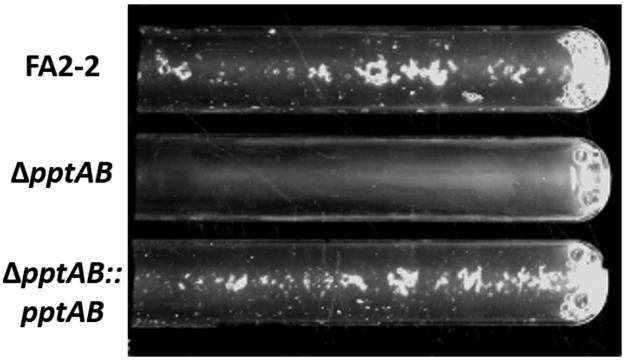

FIG 3 Clumping assay using FA2-2, FA2-2Δ*pptAB*, and FA2-2Δ*pptAB::pptAB* as recipients. Donor cells harbored the pCF10 plasmid. After the donors and recipients were incubated for 4 h under rigorous shaking conditions (200 rpm), the tubes were placed horizontally to visualize the extent of cell clumping and photographed.

The Δ*pptAB* mutant fails to aggregate in clumping assays with donor cells possessing a pheromone-responsive plasmid. Our inability to detect cOB1 in the supernatant of a *pptAB* mutant suggested that the PptAB ABC transporter might be a global pheromone exporter. A hallmark of pheromone-mediated conjugation in broth matings is the formation of cellular aggregates or "clumps" and is indicative of a coaggregation event between donor and recipient cells, recently reviewed by Clewell et al. (9). Plasmid-bearing donor cells respond to the presence of peptide pheromones produced by the recipient to induce the expression of conjugation machinery that also includes aggregation substance. Clumping occurs when aggregation substance on the donor cell binds to binding substance on the recipient to facilitate mating bridge formation and subsequent transfer of the plasmid from donor to recipient. The cell aggregation assay was performed using FA2-2, FA2-2Δ*pptAB*, and the complemented strain FA2-2Δ*pptAB*::*pptAB* as the recipient cells and OG1SSp(pCF10) as the donor cells. The transconjugation mixture containing the parental strain FA2-2 and the complemented strain showed visible clumping with the OG1SSp(pCF10) donor, while the Δ*pptAB* mutant failed to induce detectable clumping (Fig. 3), suggesting that the peptide pheromone transporter is also essential for the secretion of other peptide sex pheromones besides cOB1.

Mating efficiency with three well-characterized pheromone-responsive plasmid systems is significantly attenuated by deletion of the PptAB transporter. Since the *pptAB* mutant showed no visible clumping in the cell aggregation assay, we examined whether the mating efficiency between the donor and recipient cells was affected in a broth mating assay and extended the observations to 3 unique and well-characterized pheromone-responsive plasmid systems (pCF10, pAD1, and pAM373). The broth mating assay (25) involved (as the donor cells) OG1SSp harboring either pCF10, pAM714 (a pAD1 derivative tagged with erythromycin resistance [26]), or pAM378 (a pAM373 derivative tagged with tetracycline resistance [27]) and (as the recipient cells) FA2-2, FA2-2Δ*pptAB*, FA2-2Δ*pptAB*::*pptAB*, or (as a control) FA2-2Δ*eep* (28), as Eep was previously shown to contribute to pheromone processing in *E. faecalis* (14). The cells from each transconjugation mixture were serially diluted and plated onto medium containing antibiotics to select for transconjugants and onto a separate medium to select only for donors in order to calculate the transconjugation efficiency (number of transconjugants/number of donors). The results of the assay are shown in Fig. 4 and Table 1. We observed that in all three plasmid systems, the mating efficiency of the Δ*pptAB* mutant was significantly impaired, displaying a 5- to 6-log reduction in mating efficiency compared to that of the parent. The mating efficiency of the complemented strain was restored to wild-type levels in all three systems, whereas that of the *eep* mutant exhibited a 2- to 3-log reduction compared to that of the wild type with plasmids pCF10 and pAM714. In contrast, the *eep* mutant did not exhibit a significant change in efficiency from wild-type levels with the pAM378 plasmid, which is consistent with a previous report (14) that demonstrated a role for Eep in the generation of the cCF10 and cAD1 peptide pheromones but not of cAM373.

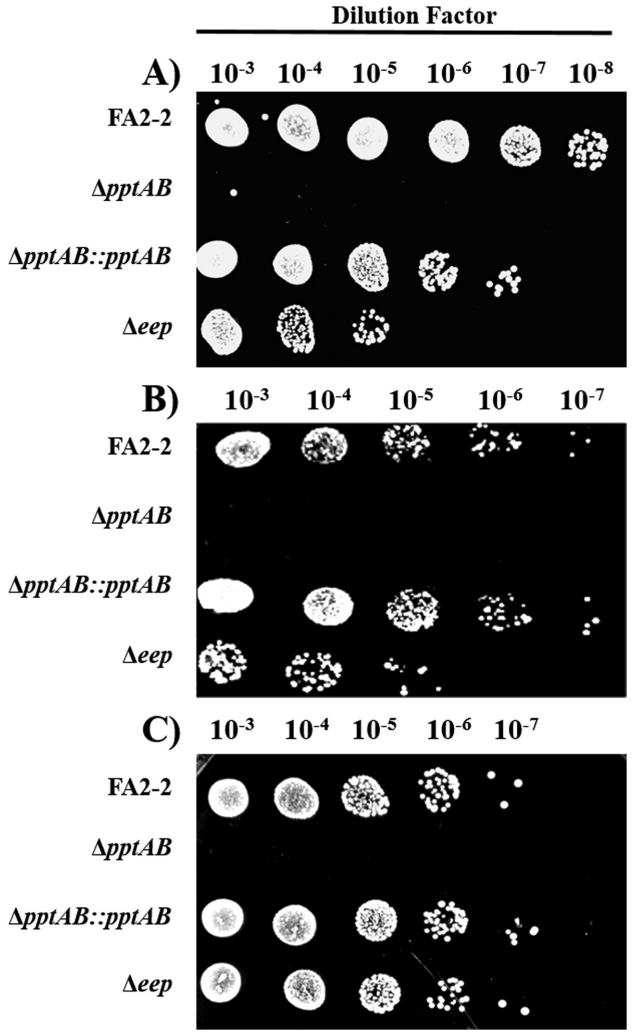

FIG 4 Broth mating assays using three different pheromone-responsive plasmid systems. Recipient strains FA2-2, FA2-2Δ*pptAB*, FA2-2Δ*pptAB::pptAB*, and FA2-2Δ*eep* were mixed with the OG1SSp donor strain possessing the pheromone-responsive plasmid pCF10 (A), pAM714 (B), or pAM378 (C). After being mixed, donors and recipients were incubated for 4 h under rigorous shaking conditions. Following a series of 10-fold dilutions, 5 μl of diluted culture was spotted onto plates with the appropriate antibiotics to select for transconjugants. Plates were incubated at 37°C overnight and photographed.

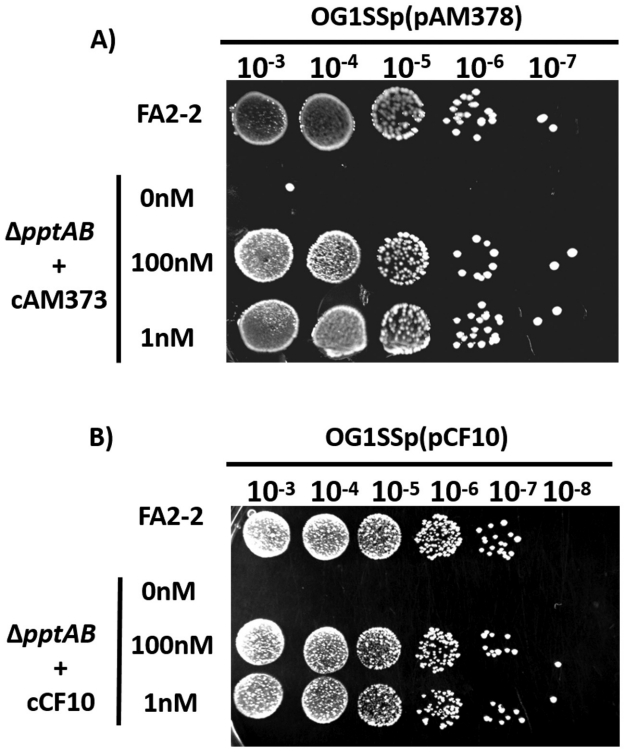

FIG 5 Exogenous addition of purified cAM373 restores the mating defect of the *pptAB* mutant. Purified cAM373 at the concentrations indicated was added to the transconjugation mixture in which FA2-2Δ*pptAB* was used as a recipient. OG1SSp(pAM378) was used as the donor. The FA2-2 parental strain was also used as a control recipient strain. Ten microliters of all reaction mixtures was serially diluted and spotted on THB agar plates containing rifampin at 10 $\mu g \cdot ml^{-1}$ and tetracycline at 10 $\mu g \cdot ml^{-1}$ to select for transconjugants.

Exogenous addition of purified sex pheromone complements the absence of the transporter. To determine whether the *pptAB* mutant was defective in cell aggregation assays and mating assays solely due to its inability to secrete peptide sex pheromones, we sought to complement the *pptAB* mutant phenotypes using synthetic pheromones. Cell aggregation assays and mating assays were performed as described above, wherein OG1SSp(pAM378) and OG1SSp(pCF10) were used as the donors and FA2-2 and FA2-2Δ*pptAB* were used as the recipients. Synthetic cAM373 and cCF10 were exogenously added to the corresponding reaction tubes containing FA2-2Δ*pptAB* recipient cells at various concentrations (1,000 nM, 100 nM, and 1 nM), and clumping was monitored. After 4 h, the cultures were plated to determine the mating efficiency. The results of these assays are shown in Fig. 5. The exogenous addition of purified cAM373 or cCF10 restored the clumping and mating defects of FA2-2Δ*pptAB* recipient cells in the cognate pheromone-responsive plasmid systems, demonstrating that the inability of the transporter mutant to actively secrete pheromones is the basis for the observed mating defects in this mutant.

TABLE 1 Transconjugation efficiency

Cell strain	Mean no. of transconjugants/no. of donor cells ± SD		
	OG1SSp(pCF10)[a]	OG1SSp(pAM714)[a]	OG1SSp(pAM378)[a]
FA2-2	$5.3 \times 10^{-1} \pm 3.6 \times 10^{-1}$	$2.3 \times 10^{-1} \pm 1.2 \times 10^{-1}$	$3.1 \times 10^{-1} \pm 1.3 \times 10^{-1}$
FA2-2Δ*pptAB*	$3.7 \times 10^{-7} \pm 0.8 \times 10^{-7}$	$1.0 \times 10^{-6} \pm 0.2 \times 10^{-6}$	$1.2 \times 10^{-6} \pm 0.3 \times 10^{-6}$
FA2-2Δ*pptAB*::*pptAB*	$9.3 \times 10^{-1} \pm 7.5 \times 10^{-1}$	$0.4 \times 10^{-1} \pm 0.3 \times 10^{-1}$	$1.2 \times 10^{-1} \pm 0.2 \times 10^{-1}$
FA2-2Δ*eep*	$5.0 \times 10^{-4} \pm 4.5 \times 10^{-4}$	$1.3 \times 10^{-3} \pm 0.2 \times 10^{-3}$	$8.6 \times 10^{-1} \pm 8.0 \times 10^{-1}$

[a] Pheromone-responsive plasmid.

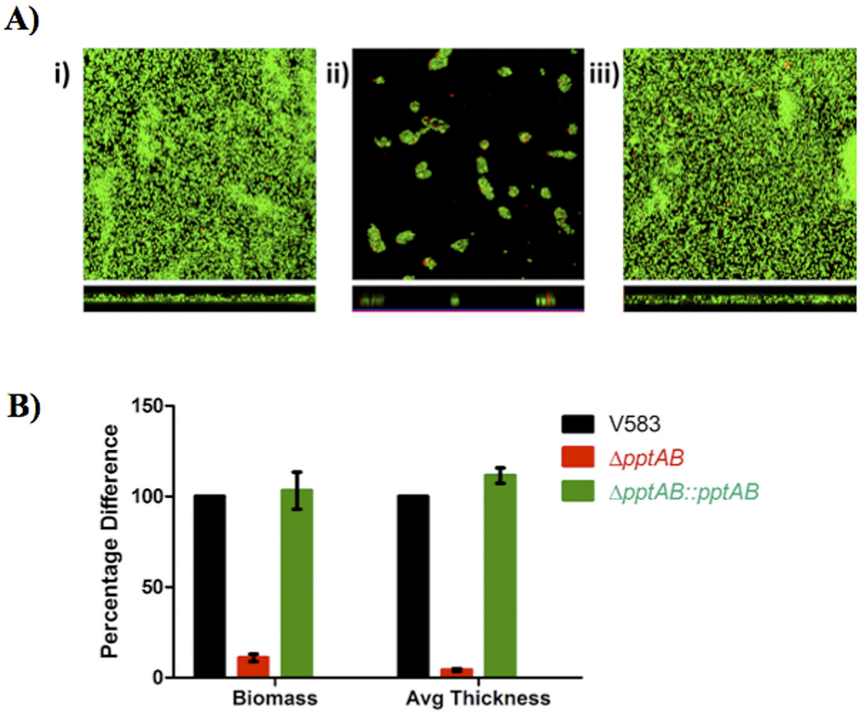

FIG 6 (A) Confocal microscopy biofilm analysis was performed on 1-day-old biofilms of V583 (i), V583Δ*pptAB* (ii), and V583Δ*pptAB*::*pptAB* (iii). All the strains contain the plasmid pMV158GFP, which provides constitutive expression of GFP. (B) COMSTAT image analysis of biofilms depicted in panel A.

Deletion of the peptide pheromone transporter affects biofilm formation. Biofilm formation is a coordinated process that generally involves cell-cell communication. Since peptide pheromones are involved in direct cell-cell signaling, we hypothesized that the deletion mutant lacking the peptide pheromone transporter would display a biofilm architecture different from that of the parental strain. To test this hypothesis, we engineered the clinical isolate *E. faecalis* V583 to delete *pptAB*, as previous studies showed that it formed better biofilms than FA2-2 (29, 30). We also complemented the V583Δ*pptAB* mutant by restoring the *pptAB* genes to their native locus using the gene knock-in strategy (23). To monitor biofilm development, plasmid pMV158GFP was introduced into each strain to provide constitutively expressed green fluorescent protein (GFP) (31). Confocal image analysis was performed on 1-day biofilms formed by V583, V583Δ*pptAB*, and the complemented strain V583Δ*pptAB*::*pptAB*. The results are shown in Fig. 6A and B. Consistently with our hypothesis, the *pptAB* transporter mutant displayed a nonconfluent biofilm phenotype compared to that of the parental and complemented strains. By COMSTAT analysis (32) (Fig. 6B), the average biofilm thickness and overall biomass were reduced by ~90% in the *pptAB* mutant compared to those in V583 and the complemented strain.

DISCUSSION

In this study, we have shown that the EcsAB homolog in *E. faecalis*, which we have renamed PptAB to reflect its apparent global role in peptide pheromone secretion, is important in the well-known cell-cell communication events in both plasmid conjugation and biofilm development. The initial studies involving EcsAB postulated that this transporter might be involved in clearing the membrane from the accumulation of peptide signals, but the nature of the transported substrate was unknown (16, 21). Here we have shown for the first time that an important substrate of the ABC transporter is the peptide signals used in pheromone mating in *E. faecalis*. The pathway by which lipoproteins are processed, leading to the production and release of pheromones, is illustrated in Fig. 7. It is known that peptide sex pheromones in *E. faecalis* are derived from lipoprotein precursors (33, 34). The lipoprotein precursors are thought to be secreted in Gram-positive bacteria through a Sec-dependent pathway (35). It is also known that the prolipoprotein diacylglyceryl-transferase (Lgt) is important in lipoprotein membrane anchorage in *E. faecalis* (36) and that the lipoprotein signal peptidase (LspA), which is a type II signal peptidase, is important for N-terminal processing of lipoproteins (37). In the present study, our transposon screen confirmed a role for LspA in the production of cOB1. In addition to these upstream processing events of the lipoprotein precursor, An et al. (14) showed that Eep also enhanced the recovery of the native pheromone. Our data extend this model further to show that the secretion of the peptide pheromones is dependent on active transport mediated by PptAB. This is supported by two important observations: (i) the addition of exogenous peptide restores the mating deficiency when the *pptAB* mutant is used as the recipient, and (ii) the peptide pheromone transporter mutant can function as a donor and efficiently transfer a pheromone-responsive plasmid to a recipient, suggesting that the inability to secrete peptide sex pheromones does not affect this mutant's ability to function as a pheromone-responsive donor (Table S5). It is important to note that an *eep* deletion mutant shows only a partial attenuation in mating efficiency compared to that of the *pptAB* deletion mutant. This may be due to the presence of a secondary protease that might compensate for the activity of Eep, as was observed with LspA and

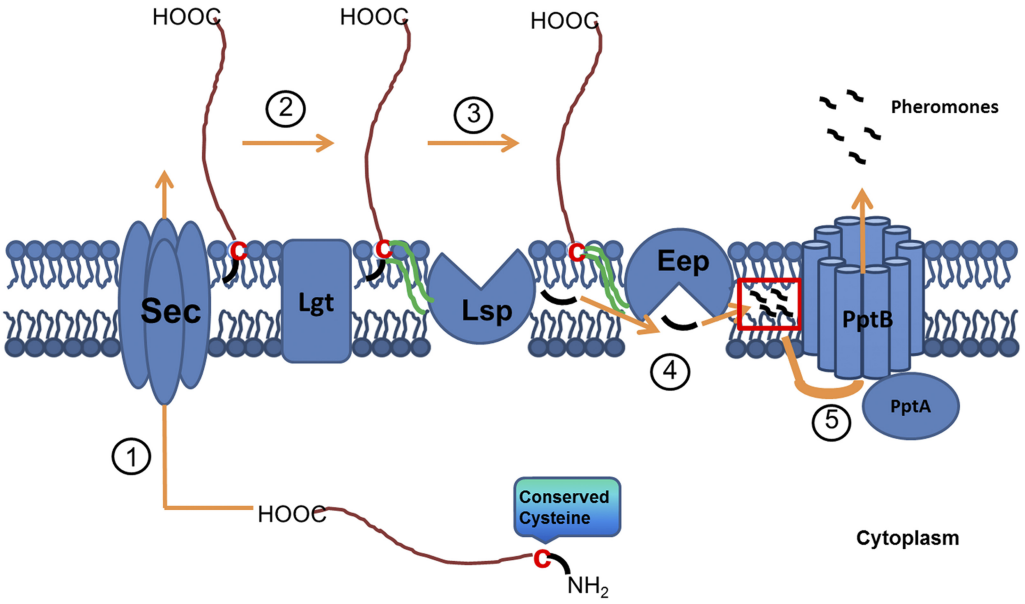

FIG 7 Mechanism of peptide sex pheromone production and secretion in *Enterococcus faecalis*. (1) Lipoprotein produced in the cytoplasm is exported out through the secretory apparatus. (2) Lgt diacylates the lipoprotein at the conserved cysteine (indicated by "C") and anchors it to the membrane. (3) Lsp, a signal peptidase 2 cleaves the N-terminal overhang from the conserved cysteine. (4) Eep, a membrane-embedded zinc metalloprotease further degrades the N-terminal overhang to produce pheromones. (5) PptAB, the peptide transporter, actively transports these pheromones outside the cell.

Eep in *Streptococcus uberis* (38). The other possibilities are that the peptide pheromone transporter might still export the prepheromones with less efficiency than the processed pheromone and that these prepheromones may retain biological activity. Confirming these hypotheses is the current focus of ongoing studies in our lab. A recent study showed that peptide pheromones mediate biofilm formation in *Streptococcus pyogenes* by interacting with the Rgg family of transcriptional regulators (39). *S. pyogenes* Rgg2 and Rgg3 bind peptide pheromones encoded by short coding sequences that are proteolytically processed to produce short hydrophobic peptide (SHP) effectors. A study by Shelburne et al. (40) identified the secretion signal sequence from the *S. pyogenes* protein Vfr as a peptide pheromone that coordinates the activity of another Rgg family member, the RopB repressor. Recent work by Dumoulin et al. (41) identified an Rgg homolog in *E. faecalis*, which they termed ElrR, as it was shown to positively regulate the expression of the enterococcal leucine-rich protein ElrA, but whether ElrR also regulates biofilm development is not presently known. It is possible that unidentified peptide pheromones of *E. faecalis* regulate biofilm formation, but whether those sequences derive from lipoprotein signal sequences, other secretion signal sequences, or additional SHP-like sequences awaits further study to determine if PptAB is directly involved in the secretion of these peptides. Deciphering the link with PptAB and biofilm formation will be valuable in understanding the role of cell-cell communication in *E. faecalis* biofilm development. Bioinformatic analysis revealed that the peptide pheromone transporter is highly conserved across the pathogenic *Firmicutes* (Fig. S3 to S4). Pheromone-mediated transfer of genetic material is a hallmark of enterococcal biology, allowing the acquisition of a variety of antibiotic resistance traits, making them more persistent pathogens in hospital settings (42), and may also disseminate resistance to other problematic pathogens. Studies have shown that clinical isolates of *Staphylococcus aureus* obtained the vancomycin resistance gene via the conjugative transposon Tn*1546* present in the coinfecting *E. faecalis* isolate (43, 44). This is attributed to the fact that *S. aureus* produces a pheromone, derived from a lipoprotein signal sequence, that is known to mimic the enterococcal pheromone cAM373, enabling exchange of genetic information between these two species (5). Recently, a genetic determinant in *Streptococcus gordonii* encoding a predicted lipoprotein was also identified as a precursor for a peptide pheromone resembling cAM373, which enables intergeneric DNA transfer between *E. faecalis* and *S. gordonii* (45). It is also interesting to note that peptide signaling has been employed by other Gram-positive organisms to perform a variety of cellular functions, including competence, sporulation, and biofilm development. In *B. subtilis*, Phr pentapeptides have been shown to be important for competence and sporulation initiation (46). In naturally transformable *Streptococcus mutans*, Sig X-regulated genes are involved in the competence cascade, and Sig X is regulated by ComR. ComR is a transcriptional regulator of the Rgg family, which is known to bind peptides derived from ComS (XIP). The XIP peptides are thought to be exported through an unidentified membrane transporter (47). More recently, Chang et al. showed that SHP pheromones, which affect virulence in *S. pyogenes* by binding Rgg, are processed by Eep and are transported outside the cell by an unknown transporter (39). Phr, XIP, and SHP pheromones are hydrophobic in nature, as are the peptide pheromones that mediate conjugation in *E. faecalis*. Based on the observations that we report in this study and the conservation of the PptAB (EcsAB) ABC transporter among pathogenic Gram-positive organisms, it is coherent to speculate that this transporter might serve the same important peptide secretory function in these pathogenic species as in *E. faecalis*. Further characterization of this conserved transporter in these pathogenic bacteria is warranted.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Pertinent bacterial strains and plasmids used in the current study are listed in Tables S2 and S3 in the supplemental material. Strains were cultured in Todd-Hewitt broth (THB) and grown at 37°C unless otherwise indicated. *Escherichia coli* Electro-Ten Blue (Stratagene, La Jolla, CA) was used for maintenance and propagation of plasmid constructs. Antibiotics for selection included chloramphenicol at 10 $\mu g \cdot ml^{-1}$, rifampin at 100 $\mu g \cdot ml^{-1}$, tetracycline at 10 $\mu g \cdot ml^{-1}$, erythromycin at 10 $\mu g \cdot ml^{-1}$, spectinomycin at 500 $\mu g \cdot ml^{-1}$, and streptomycin at 500 $\mu g \cdot ml^{-1}$. Transformation of *E. faecalis* was done as described previously (48).

Transposon mutagenesis. A mutant transposon library was constructed in *E. faecalis* strains FA2-2 and OG1RF using the transposon mutagenesis vector pCAM45 (17). Electrocompetent *E. faecalis* cells were prepared as described previously (49). Briefly, pCAM45 (1 μg) was electroporated into *E. faecalis* strains (2.5 kV, 200 Ω, 25 μF) in 0.2-cm electroporation cuvettes. Cells were allowed to recover for 2 h at 30°C in 1 ml SGM17MC recovery medium before being plated on THB containing erythromycin (50 $\mu g \cdot ml^{-1}$) and kanamycin (2,000 $\mu g \cdot ml^{-1}$). Plates were incubated for 48 to 72 h, and the resulting colonies were grown in THB supplemented with erythromycin at 20 $\mu g \cdot ml^{-1}$. Following overnight growth at 30°C, cells were diluted 1:100 into fresh THB and incubated for 2.5 h at 30°C and then shifted to 42°C for 2 h. After the 42°C incubation step, cells were serially diluted and plated onto prewarmed THB plates containing 20 $\mu g \cdot ml^{-1}$ erythromycin and incubated at 42°C overnight to obtain the transposon mutant library.

Screen for inhibitory activity against V583. To identify the OG1RF and FA2-2 mutants that no longer possessed the inhibitory activity against V583, a growth inhibition bioassay was performed. Briefly, 250 μl of an overnight culture of V583 was suspended in 7.5 ml of THB soft agar. The soft agar suspension was laid over THB agar containing either the OG1RF or the FA2-2 transposon mutant plated at a density of 200 to 300 colonies per plate. Colonies that failed to exhibit inhibitory activity against V583 were picked onto a THB agar plate containing rifampin at 100 $\mu g \cdot ml^{-1}$ and fusidic acid at 50 $\cdot$ μg ml^{-1} to select for either OG1RF or FA2-2 derivatives for further characterization.

Mapping of transposon integration sites. To map the location of the transposon in the mutants identified in the V583 killing screen, we sequenced the flanking DNA by amplifying the region surrounding the transposon insertion via PCR with a primer specific to one end of the Himar 1 transposon (VT01) and an arbitrary primer (VT02) with a constant region at the 5′ end. In a second round of PCR, a nested primer (VT04) specific to the Himar 1 transposon was used with a primer specific to the constant region of the arbitrary primer (VT03). The resulting product was sequenced by using a third nested primer (VT05) specific to the transposon, as described previously (50). Briefly, a colony from the transposon mutant was suspended in15 μl of doubly distilled water (ddH_2O). Two microliters of the suspension was added to 48 μl of a PCR master mix containing 5 μl of 10× *Taq* buffer, 1 μl of 50 mM $MgCl_{24}$, μl deoxynucleoside triphosphates (dNTPs) at 2.5 mM, 1 μl primer VT01, 1 μl primer VT02, 35.8 μl of ddH_2O, and 0.2 μl of *Taq* polymerase (1 unit). Following the 1st round of PCR, products were diluted 1:25, and 5 μl of the diluted suspension was added to 45 μl of a master mix containing 5 μl of 10× *Taq* buffer, 1 μl of 50 mM $MgCl_{24}$, dNTPs at 2.5 mM, 1 μl primer VT04, 1 μl primer VT03, 35.8 μl of ddH_2O, and 0.2 μl of *Taq* polymerase (1 unit). To sequence the resulting PCR products, primer VT05 was used.

Construction of an *E. faecalis pptAB* in-frame deletion mutant. In-frame deletion of *pptAB* was performed using a plasmid derived from pLT06 (51). Briefly, flanking regions (~1 kb) from both the 5′ and 3′ ends of the *pptAB* ABC transporter were PCR amplified by using the primers listed in Table S3. For the construction of the pSV02 plasmid (*pptAB* deletion), the primers PptABP1 and PptABP2 were used to amplify the region 5′ of the translation start site of *pptA* on the V583 genome. Primers PptABP3 and PptABP4 were used to amplify the region 3′ of the translation stop site of *pptB*. The PptABP1 and PptABP2 region contained EcoRI and BamHI sites, and PptABP3 and PptABP4 contained BamHI and SphI sites. Each product was cut with BamHI, religated, and reamplified with primers PptABP1 and PptABP4 to obtain an amplicon with the *pptAB* genes deleted. This amplicon was digested with EcoRI and SphI and ligated into similarly digested pLT06. The insert and vector were ligated and electroporated into competent *E. coli* ElectroTen-Blue cells (Stratagene, La Jolla, CA). Constructs were screened by colony PCR, and positive clones were further confirmed by restriction mapping and DNA sequencing. The plasmid construct containing the deletion construct for *pptAB*, designated pSV02, was electroporated into electrocompetent *E. faecalis* FA2-2 or V583 cells (49). SV02 (V583Δ*pptAB*) and SV04 (FA2-2Δ*pptAB*) were generated by following the protocol previously described (52). Mutants were confirmed by PCR using the primers PptABUp and PptABDown.

Complementation of *E. faecalis* FA2-2 and V583 in-frame *pptAB* deletion mutants. The in-frame *pptAB* deletion mutations in FA2-2 and V583 were complemented by using a previously described pLT06 vector system based on a knock-in strategy (23). For the construction of the complementation vector pSV06, primers PptABP1 and PptABP4 were used to amplify an ~4-kb region containing the *pptAB* gene and the ~1-kb flanking regions on either side from V583 genomic DNA. This product was then cut with EcoRI and SphI and ligated into pLT06 cut with the same enzymes. The remaining steps were done similarly to the procedures described above. Strains possessing the complemented *pptAB* genes were designated SV06 (FA2-2Δ *pptAB*::*pptAB*) and SV18 (V583Δ *pptAB*::*pptAB*), respectively.

Cell aggregation assay. Clumping activity was assayed using a protocol modified from the work of Mori et al. (25). *E. faecalis* OG1SSp cells containing the different pheromone-responsive plasmids pCF10, pAM714 (a pAD1 plasmid derivative), and pAM378 (a pAM373 plasmid derivative) were used as the donor cells. Wild-type *E. faecalis* FA2-2, the Δ*pptAB* mutant (SV02), and the complemented strain (SV06) were used as the recipients. Briefly, the donor cells and the recipient cells were grown overnight at 37°C in 2 ml of THB. Fifty microliters of each donor cell was added to 450 μl of either FA2-2, SV04, or SV06, and this mixture was added to 4.5 ml of fresh THB medium. The cultures were grown at 37°C in a shaking incubator (~200 rpm) for 4 h to visually compare the differences in clumping phenotype.

Transconjugation assay. Transconjugation efficiency was calculated using a mating assay modified from the work of Mori et al. (25). After incubation of the donor and recipient cells together for 4 h under shaking conditions (200 rpm), the cell cultures from the different plasmid systems were serially diluted in sterile phosphate-buffered saline (PBS) and track diluted (53) onto THB containing rifampin at 100 $\mu g \cdot ml^{-1}$ and tetracycline at 15 $\mu g \cdot ml^{-1}$ (for pCF10 and pAM378 transconjugants) or THB containing rifampin at 100 $\mu g \cdot ml^{-1}$ and erythromycin at 10 $\mu g \cdot ml^{-1}$ (for pAM714). The cultures were also track diluted onto THB plates containing spectinomycin at 500 $\mu g \cdot ml^{-1}$ and streptomycin at 500 $\mu g \cdot ml^{-1}$ to select for donor cells in all three plasmid systems. Transconjugation efficiency was measured as the ratio of transconjugants to donor cells. For the exogenous addition of cAM373 and cCF10, a 1 mM stock solution of chemically synthesized cAM373 was prepared in 50% acetonitrile solution and a 1 mM stock of cCF10 was prepared in dimethyl formamide; these were serially diluted to obtain the working concentrations. Working stocks were added to the transconjugation reactions as indicated in Fig. 5.

Confocal biofilm analysis. Confocal laser scanning microscopy (CLSM) was performed on 1-day-old biofilms as described previously (30). *E. faecalis* strains SV02 and SV18 were transformed with pMV158GFP to generate SV20 and SV23, respectively, as both these strains expressed GFP constitutively. VT09 [V583(pMV158GFP)] (30) along with SV20 and SV23 were used for confocal microscopy analysis. Briefly, biofilms were grown on sterile glass coverslips placed in six-well tissue culture plates. Five milliliters of TSB containing tetracycline was added to the wells containing the coverslip for plasmid maintenance. After 24 h of growth, the biofilms were gently washed with sterile PBS

(PBS137; mM NaCl, 2.7 mM KCl, 10 mM Na2 HPO_{42}, mM KH2 PO_4 [pH 7.4]) and stained with 1 μM Sytox orange (Invitrogen) for 6 to 7 min. The coverslips were inverted on a clean glass slide and sealed using clear nail polish. The biofilm was visualized using a 5-Pa Zeiss laser scanning confocal microscope.

Growth curve analysis. Standard growth curve analysis was performed to ensure that SV04 (Δ*pptAB*) and SV06 (*pptAB* complementation) did not exhibit growth defects relative to the growth of the parental FA2-2 strain. Briefly, FA2-2, SV04, and SV06 were grown in THB overnight at 37°C. The overnight cultures of these strains were inoculated into 50-ml tubes containing sterile THB at a 1:100 ratio and were incubated at 37°C. Samples from each tube were aseptically withdrawn every hour, and the optical density was measured at a wavelength of 600 nm through stationary-phase growth (~8 h). The skim milk plate assay was performed by spotting 5 μl of an overnight culture of FA2-2, OG1RF, OG1RF::EF_0689a and OG1RF::EF_0689b onto a THB plate containing 1.5% skim milk. The plate was incubated overnight at 37°C, and the zone of clearance was observed.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01726-14/-/DCSupplemental.

Text S1, PDF file, 0.2 MB.
Figure S1, PDF file, 0.4 MB.
Figure S2, PDF file, 0.4 MB.
Figure S3, PDF file, 0.5 MB.
Figure S4, PDF file, 0.5 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.2 MB.
Table S3, PDF file, 0.1 MB.
Table S4, PDF file, 0.2 MB.
Table S5, PDF file, 0.2 MB.

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